The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway

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Interleukin-1 (IL-1) is a proinflammatory cytokine that has several effects in the inflammation process. When it binds to its cell-surface receptor, IL-1 initiates a signalling cascade that leads to activation of the transcription factor NF-κB and is relayed through the protein TRAF6 and a succession of kinase enzymes, including NF-κB-inducing kinase (NIK) and IκB kinases (IKKs)[1–7]. However, the molecular mechanism by which NIK is activated is not understood. Here we show that the MAPKK kinase TAK1 (ref. 8) acts upstream of NIK in the IL-1-activated signalling pathway and that TAK1 associates with TRAF6 during IL-1 signalling. Stimulation of TAK1 causes activation of NF-κB, which is blocked by dominant-negative mutants of NIK, and an inactive TAK1 mutant prevents activation of NF-κB that is mediated by IL-1 but not by NIK. Activated TAK1 phosphorylates NIK, which stimulates IKK-α activity. Our results indicate that TAK1 links TRAF6 to the NIK–IKK cascade in the IL-1 signalling pathway.

After binding to the cell-surface type-I IL-1 receptor (IL-1RI), IL-1 triggers a cascade of signalling events, including activation of c-Jun N-terminal kinase (JNK) and of NF-κB, which upregulates the expression of many proinflammatory genes in the nucleus[9]. NF-κB is rendered inactive in the cytoplasm by inhibitory proteins called IκB in unstimulated cells. In response to extracellular stimuli, the IκB proteins are phosphorylated on specific serine residues and rapidly degraded, leading to the nuclear localization and activation of NF-κB[10–12]. A kinase complex consisting of NIK and IKK-α/β is involved in signal-induced phosphorylation of IκB proteins[2–7]. When IL-1 binds to IL-1RI, IL-1RI forms a complex with an IL-1-receptor accessory protein, resulting in the recruitment of MyD88 and of the Ser/Thr kinase IRAK to the receptor. IRAK then dissociates from the receptor complex and interacts with TRAF6, which transduces the IL-1 signal to the NIK-IKK-α/β kinase pathway[1,13,14]. TRAF6 has been implicated in the activation of both JNK and NF-κB[15]. In contrast, NIK is essential for IL-1-mediated NF-κB activation but has no effect on the activation of JNK[15,16]. Thus, the molecular mechanism linking TRAF6 to the activation of NIK is likely to involve additional signalling molecules. TAK1 is a recently identified MAP kinase kinase kinase (MAP3K) that can activate JNK[8,17,18]; another protein, TAB1, functions as an activator of TAK1 (ref. 19). We now examine the role of TAK1 in the NIK–IKK cascade that operates in the IL-1 signalling pathway.

Treatment of 293IL-1RI cells with IL-1 resulted in activation of endogenous TAK1 in a time-dependent manner (Fig. 1a). To determine whether TAK1 participates in IL-1 signalling, we investigated the interaction of TAK1 and TAB1 with TRAF6. 293IL-1RI cells were either treated with IL-1 or left untreated; lysates were immunoprecipitated with antibody against TRAF6 and the co-precipitated TAK1 and TAB1 were detected by immunoblot analysis (Fig. 1b). Endogenous TAK1 and TAB1 were found to associate with TRAF6 in IL-1-treated cells but not in untreated cells. Thus, the association of endogenous TRAF6 with TAK1 and TAB1 is ligand-dependent. Next, 293 cells were transiently transfected with an expression vector encoding a Flag-epitope-tagged TRAF6 (Flag–TRAF6) protein. Immunoprecipitation of Flag–TRAF6 caused co-precipitation with TAK1 and TAB1 even from lysates of cells not treated with IL-1 (Fig. 1c). This indicates that TRAF6 constitutively associates with TAB1 and TAK1 when TRAF6 proteins are over-

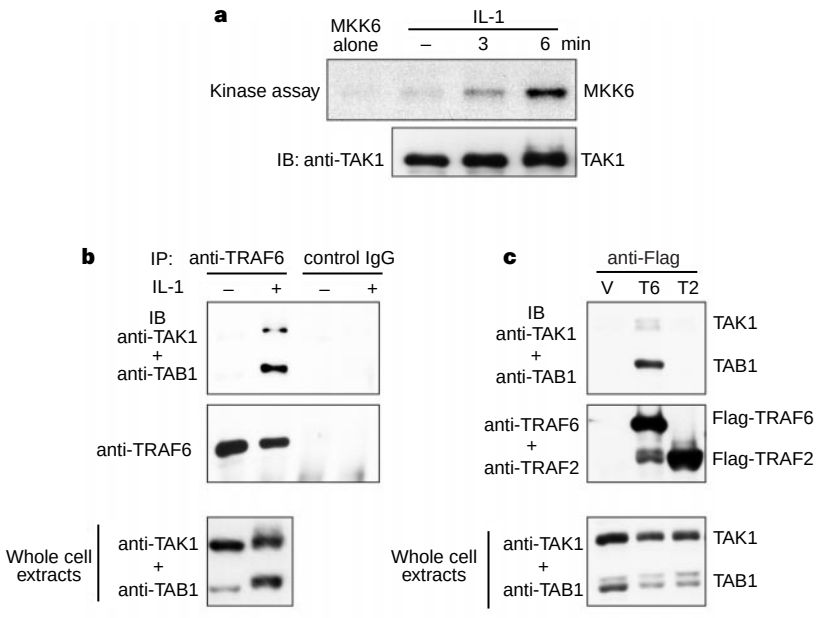

Figure 1 Effect of IL-1 on TAK1. **a**, IL-1-induced activation of TAK1 activity. 293IL-1RI cells were treated with IL-1 (10 ng ml^{-1}). Immunoprecipitated complexes with anti-TAK1 were subjected to kinase reactions with MKK6 and immunoblotted (IB) with anti-TAK1. **b**, **c**, Association of TRAF6 with TAK1. 293IL-1RI cells were stimulated for 10 min with IL-1 or left untreated. Cell lysates were immunoprecipitated (IP) with anti-TRAF6 or IgG (**b**). 293 cells were transfected with empty vector (V), Flag–TRAF6 (T6), or Flag-TRAF2 (T2). Cell lysates were immunoprecipitated with anti-Flag (**c**). The immunoprecipitates were immunoblotted with each antibody. Expression of TAK1 and TAB1 was monitored.

expressed and is consistent with the observation that TRAF6 overexpression can activate NF-κB in the absence of ligand[1]. We also investigated the interaction of TAK1 and TAB1 with another member of the TRAF family, TRAF2, which has been implicated in the TNF-mediated signalling pathway[15]. In contrast to TRAF6, TAB1–TAK1 did not co-precipitate with TRAF2 complex (Fig. 1c).

TRAF6 has been implicated in IL-1-induced NF-κB activation[1]. To determine whether TAK1 also functions in the activation of NF-κB, we tested the effects of activation of TAK1 on the expression of an NF-κB-dependent reporter gene in transiently transfected 293 cells. Co-expression of TAB1 and TAK1 activated the kinase activity of TAK1 and increased transcription of the reporter gene (Fig. 2a). Overexpression of another member of the MAP3K family, MTK1 (ref. 20), failed to induce NF-κB activation (data not shown). NF-κB was not activated when TAB1 and a kinase-inactive mutant of TAK1, TAK1(K63W), were co-expressed, indicating that TAK1-mediated NF-κB activation is dependent on its kinase activity. Activation of NF-κB by IL-1 requires the successive action of NIK and IKK-α/β[2–7]. To determine the signalling pathways that couple TAK1 to NF-κB activation, we tested whether dominant-negative forms of NIK could block signalling from TAB1–TAK1. The inactive NIK mutant NIK(KK429-430AA) behaves as a dominant-negative inhibitor of IL-1-induced NF-κB activation[2]. Expression of NIK(KK429-430AA) blocked the activation of NF-κB-dependent gene expression by TAB1–TAK1 (Fig. 2b). Furthermore, in contrast to a recent observation[21], another dominant-negative form, NIK(624–947), also inhibited TAB1–TAK1-induced activation of NF-κB (Fig. 2b). Overexpression of the inactive TAK1(K63W) mutant inhibited IL-1-induced activation of NF-κB in a dose-dependent manner, but had little effect on NIK-induced activation of NF-κB (Fig. 2b). Taken together, these results indicate that TAB1–TAK1 may function upstream of the NIK–IKK cascade to mediate NF-κB activation by IL-1.

In addition to NF-κB activation, IL-1 induces activation of JNK[9]. To test whether TAK1 is involved in IL-1-induced JNK activation, we did JNK assays by using a glutathione *S*-transferase fusion protein (GST–c-Jun) as substrate. Overexpression of TAK1(K63W) blocked IL-1-induced JNK activation (Fig. 2c), suggesting that TAK1 participates in both NF-κB and JNK activation by IL-1. In contrast, the expression of NIK(KK429-430AA) at levels that gave maximal inhibition of NF-κB activation did not impair the ability of either IL-1 or TAB1–TAK1 to activate JNK (Fig. 2c). Thus, bifurcation of the IL-1-induced JNK and NF-κB activation pathways occurs at TAK1.

Our results raised the possibility that TAK1 could function as the kinase that activates NIK in the IL-1 signalling pathway. We therefore investigated whether activation of TAK1 induces phosphorylation of NIK. Immunoblot analysis of NIK from cells overexpressing wild-type NIK revealed that NIK migrated as a doublet (Fig. 4b, lane 6), possibly as a result of autophosphorylation and/or phosphorylation by activated IKK-α (ref. 3). When expressed alone, NIK(KK429-430AA) also appeared as a doublet, but with a minor form that migrated more slowly than the major form (Fig. 3a, lane 1). IL-1 treatment caused an accumulation of the more slowly migrating form of NIK (Fig. 3a, lane 2). This slower band was eliminated by phosphatase treatment (Fig. 3a, lane 7), suggesting that it represents phosphorylated NIK. When TAK1 was activated by co-expression with TAB1, the amount of slower NIK(KK429-430AA) increased (Fig. 3a, lane 5). This modification was not seen in 293 cells transfected with MTK1 MAP3K (data not shown), indicating that NIK modification is specific for TAK1. Furthermore, expression of the dominant-negative mutant TAK1(K63W) inhib-

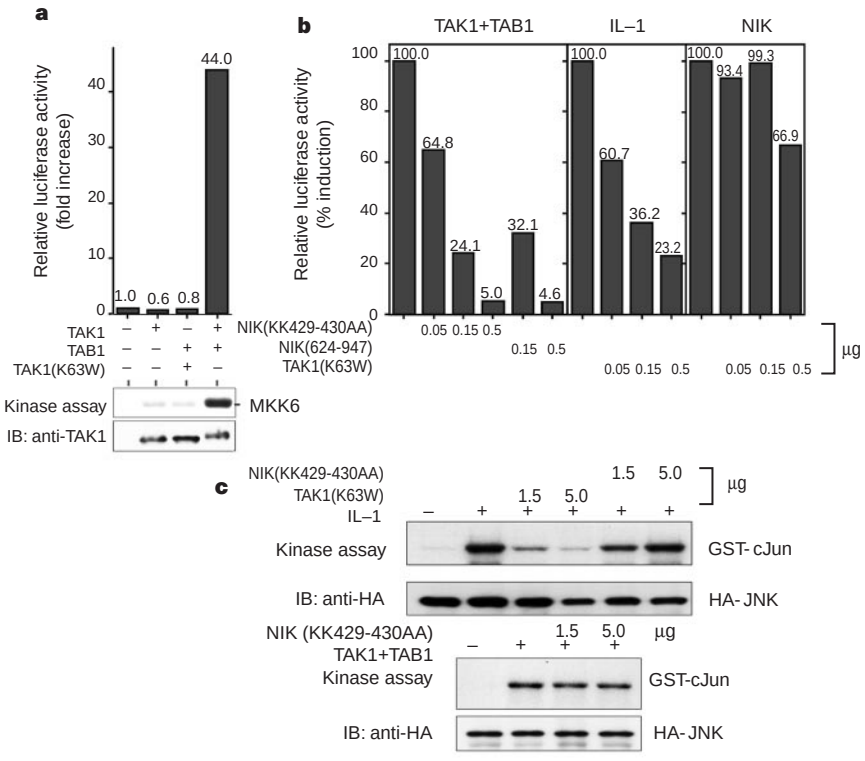

Figure 2 Effect of TAK1 on NF-κB and JNK activation. **a**, **b**, Effect of TAK1 on NF-κB activation. 293 cells were transfected with HA–TAK1, HA–TAK1(K63W) and TAB1, as indicated. Immunoprecipitated complexes with anti-HA were subjected to kinase reactions and immunoblotted with anti-TAK1 (**a**, lower panel). Ig-κ-luciferase plasmid was transfected into 293 cells together with expression vectors, as indicated. Cells were either treated with IL-1 (10 ng ml^{-1}) or left untreated. Relative luciferase activity was measured. Data are expressed as the fold increase (**a**) or the percentage induction (**b**). **c**, Effect of TAK1 on JNK activation. HA–JNK1 was transfected into 293IL-1RI cells together with expression vectors, as indicated. Cells were either treated with IL-1 for 10 min or left untreated. Immunoprecipitated complexes with anti-HA were subjected to kinase reactions with GST–cJun and immunoblotted with anti-HA.

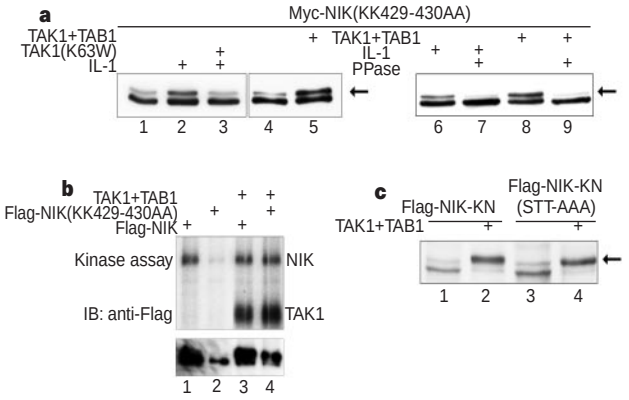

Figure 3 Phosphorylation of NIK. **a**, **c**, Effect of TAK1 on phosphorylation of NIK. 293 cells were transfected with Myc–NIK(KK429-430AA) (**a**), and Flag–NIK(KK429-430AA) (Flag–NIK-KN) or Flag–NIK(KK429-430AA;STT549,552,559AAA) [Flag–NIK-KN(STT-AAA)] (**c**). Cells were also transfected with TAK1 and TAB1 or TAK1(K63W), as indicated. Cells were treated for 30 min with IL-1 (10 ng ml^{-1}) or left untreated. Cell lysates were incubated with protein phosphatase (PPase). Anti-Myc (**a**) or anti-Flag (**c**) was used to detect NIK in total lysates. Arrows indicate the slowly migrating form of NIK. **b**, *In vitro* phosphorylation of NIK by TAK1. 293 cells were transfected with expression vectors as indicated. Complexes immunoprecipitated with anti-Flag were incubated with [γ-^{32}P]ATP and analysed by autoradiography. The migration positions of NIK and TAK1 are indicated.

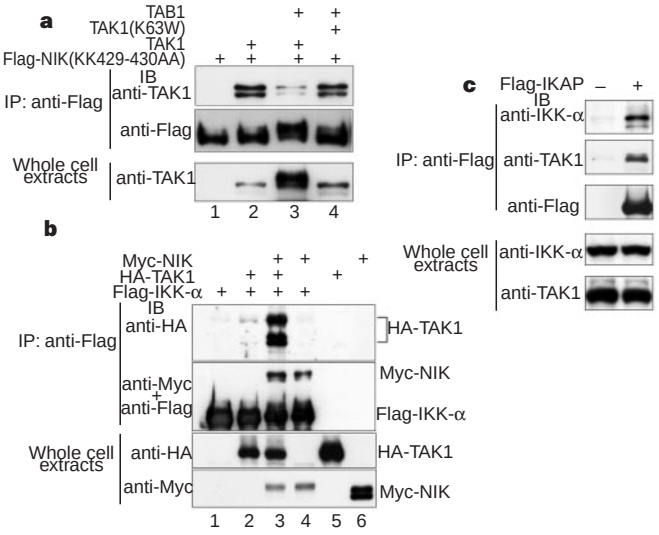

Figure 4 Interaction of TAK1 with components of the IKK complex. 293 cells were transfected with expression vectors as indicated. Cell lysates were immunoprecipitated with anti-Flag. **a**–**c**, Immunoprecipitates were immunoblotted with anti-TAK1 and anti-Flag (**a**), anti-HA, anti-Myc and anti-Flag (**b**), and anti-IKK-α, anti-TAK1 and anti-Flag (**c**). Immunoprecipitated HA–TAK1 presented as multiple bands, probably generated by phosphorylation by NIK and/or IKK-α (**b**). The expression of TAK1 (**a**), HA–TAK1 and Myc–NIK (**b**), and of IKK-α and TAK1 (**c**) was monitored.

ited IL-1-induced modification of NIK (Fig. 3a, lane 3). These findings indicate that IL-1 signalling activates TAK1, causing phosphorylation of NIK.

As activation of TAK1 resulted in NIK phosphorylation, we investigated whether TAK1 can phosphorylate NIK *in vitro* (Fig. 3b). We transiently expressed Flag-epitope-tagged wild-type NIK (Flag–NIK), or the inactive mutant Flag–NIK(KK429-430AA) in 293 cells. Epitope-tagged proteins were immunoprecipitated with anti-Flag antibody, and incubated with [γ-^{32}P]ATP. In these assays, wild-type NIK was autophosphorylated (Fig. 3b, lane 1), whereas NIK(KK429-430AA) was not (Fig. 3b, lane 2). When TAK1 and TAB1 were co-expressed with Flag–NIK(KK429-430AA), activated TAK1 phosphorylated NIK(KK429-430AA) and it became autophosphorylated *in vitro* (Fig. 3b, lane 4).

Phosphorylation of Ser and/or Thr residues in the kinase-activation loop (between subdomains VII and VIII) is essential for activation of many protein kinases[22]. There is a serine (residue 549) and two threonines (residues 552 and 559) in the activation loop of NIK (ref. 2). We generated a mutant NIK, NIK(ST-T549,552,559AAA), in which Ser 549, Thr 552 and Thr 559 were replaced with alanine residues. In agreement with recent results[23], NIK(STT549,552,559AAA) prevented NIK from activating NF-κB and functioned as a dominant-negative inhibitor of IL-1-mediated activation of NF-κB, like the NIK(KK429-430AA) mutant (data not shown). Thus, residues in the activation loop of NIK are critical for the regulation of NIK. To test whether TAK1 participates in the phosphorylation of residues within the activation loop of NIK, we generated a Flag-epitope-tagged NIK mutant, Flag–NIK(KK429-430AA;STT549,552,559AAA), in the background of the inactive NIK(KK429-430AA) mutation in order to eliminate NIK autophosphorylation activity (Fig. 3c). When TAK1 was activated by co-expression with TAB1, the NIK(KK429-430AA;STT549,552,559AAA) mutant form was still modified (Fig. 3c, lane 4), indicating that TAK1 can induce phosphorylation of NIK residues apart from the Ser 549, Thr 552 and Thr 559 residues in the activation loop.

To determine whether TAK1 associates with NIK, we co-transfected TAK1 and Flag–NIK(KK429-430AA) into 293 cells and then immunoprecipitated the lysates (Fig. 4a). TAK1 was detected in NIK(KK429-430AA) immunoprecipitates (Fig. 4a, lane 2). We also did the same experiment using cells in which TAB1 was co-expressed as well in order to activate TAK1. Although co-expression of NIK(KK429-430AA), TAB1 and TAK1 caused phosphorylation of NIK(KK429-430AA), the association of NIK(KK429-430AA) and TAK1 decreased compared with cells containing non-activated TAK1 (Fig.. 4a, lane 3). Thus, the inactive form of TAK1 preferentially associates with NIK(KK429-430AA). Alternatively, TAB1 competes with NIK for binding to TAK1. To exclude this possibility, we examined the interaction between NIK and inactive TAK1(K63W) in the presence of TAB1. TAK1(K63W) associated with NIK(KK429-430AA) more efficiently than did wild-type TAK1 (Fig. 4a, lane 4). Wild-type and inactive TAK1 associated with TAB1 to the same extent (data not shown). These results indicate that, once activated, TAK1 phosphorylates NIK and it may then be released from the NIK–IKK complex.

We next investigated whether TAK1, NIK and IKK-α are present in the same complex by using co-transfection and co-immunoprecipitation methods. Flag-epitope-tagged IKK-α (Flag–IKK-α) was transiently co-expressed in 293 cells together with either Myc-epitope-tagged NIK (Myc–NIK) or haemagglutinin-epitope-tagged TAK1 (HA–TAK1). Cell lysates were immunoprecipitated with anti-Flag antibody and analysed by immunoblotting using anti-Myc or anti-HA antibodies to detect NIK or TAK1, respectively (Fig. 4b). In this assay, NIK co-precipitated with IKK-α (Fig. 4b, lane 4), whereas TAK1 interacted only weakly with IKK-α (Fig. 4b, lane 2). To determine whether NIK can mediate interaction between TAK1 and IKK-α, we co-expressed the three proteins in 293 cells. There was a strong interaction between TAK1 and IKK-α (Fig. 4b, lane 3), indicating that TAK1 interacts with a complex consisting of NIK and IKK. Consistent with this, we found that TAK1 also associated with IKAP (IKK-complex-associated protein) (Fig. 4c). IKAP acts as a scaffold that assembles IKKs with NIK[24]. Flag-epitope-tagged IKAP (Flag–IKAP) was transfected into 293 cells and the lysates were then immunoprecipitated. Endogenous TAK1 and IKK-α were detected in IKAP immunoprecipitates.

These results indicate that activated TAK1 stimulates the kinase activity of NIK by phosphorylation. To test whether TAK1 stimu-

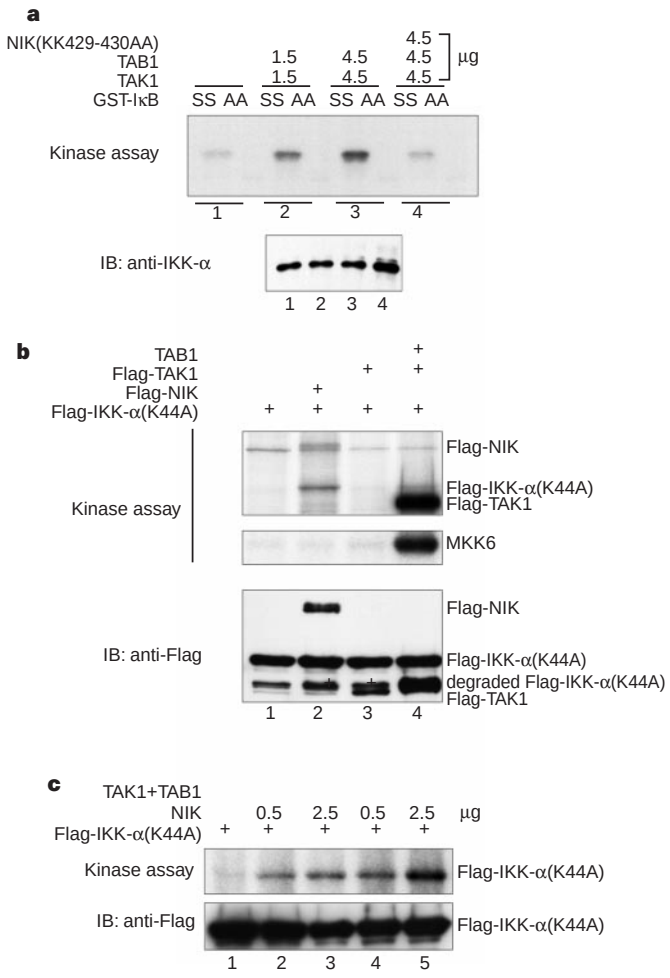

Figure 5 Activation of IKK-α by TAK1. **a**, TAK1-induced activation of IKK-α through activation of NIK. 293 cells were transfected with expression vectors as indicated. Endogenous IKK-α was immunoprecipitated with anti-IKK-α and subjected to kinase reactions with GST–IκBN (SS) or mutant GST–IκBN(S32A,S36A) (AA). **b**, **c**, *In vitro* phosphorylation of IKK-α. 293 cells were transfected with expression vectors as indicated. Immunoprecipitated complexes with anti-Flag were incubated with [γ-^{32}P]ATP and analysed by autoradiography. Immunoprecipitates were also subjected to kinase reactions with MKK6 (**b**). The positions of IKK-α(K44A), NIK and TAK1 are indicated. pcDNA3 vector was used for transfection in order to achieve low NIK expression (**c**).

lates IKK-α by activating NIK, we used an *in vitro* phosphorylation assay (Fig. 5a). Immunoprecipitates of endogenous IKK-α weakly phosphorylated IκB-α on serine residues 32 and 36 (Fig. 5a, lane 1). When TAK1 and TAB1 were co-expressed, phosphorylation of IκB-α increased in a dose-dependent manner (Fig. 5a, lanes 2, 3). This stimulation of endogenous IKK-α activity by TAB1 and TAK1 was dependent on NIK's being active; it did not occur when TAB1 and TAK1 were co-expressed with NIK(KK429–430AA) (Fig. 5a, lane 4).

NIK is a MAP3K-related kinase that phosphorylates Ser 176 during the activation of IKK-α (ref. 25). MAP3K enzymes activate MAP2K enzymes by phosphorylating serine and threonine residues in the activation loop. TAK might therefore phosphorylate IKK, so we co-expressed the tagged inactive IKK-α mutant Flag-IKK-α(K44A) with TAK1 and TAB1 or NIK (Fig. 5b), immunoprecipitated the epitope-tagged proteins and incubated them with [γ-^{32}P]ATP. NIK phosphorylated IKK-α (Fig. 5b, lane 2) as expected[25], whereas TAK1 barely phosphorylated IKK-α (Fig. 5b, lane 4). When incubated with bacterially expressed MKK6, TAK1 but not NIK phosphorylated MKK6: thus, TAK1 and NIK have different substrate specificities. When we co-transfected Flag–IKK-α(K44A), TAK1 and TAB1 with small amounts of NIK, and measured the phosphorylation of IKK-α(K44A), we found that TAK1 stimulated the phosphorylation of IKK-α(K44A) by NIK (Fig. 5c). Taken together, these results indicate that activated TAK1 promotes the phosphorylation of NIK and hence activation of IKK by NIK.

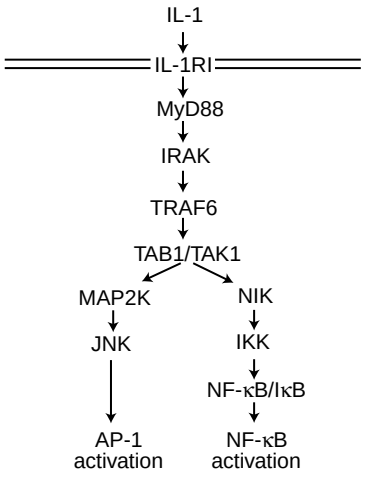

Figure 6 Model for the role of TAK1 in the IL-1 signalling pathway. See text for details.

Our results identify TAK1 as a kinase that links TRAF6 to the NIK–IKK cascade in the IL-1 signalling pathway (Fig. 6). TRAF6 also interacts with IRAK and NIK (refs 1, 15), so it may serve as a docking site for these signalling molecules in the pathway. TAK1 is recruited to TRAF6 in response to IL-1 stimulation, which may activate TAK1 and thus NIK. It is still unclear how TAK1 is activated: additional components may needed. NIK shares homology with MAP3K (ref. 2). It would be rather unusual for TAK1 MAP3K to phosphorylate another MAP3K-like kinase like NIK, although there is no direct evidence yet that NIK functions as a MAP3K. Although NIK phosphorylates IKK-α *in vitro*[25], IKK-α is not homologous to MAP2K (refs 3–5). Furthermore, NIK is not involved in activation of JNK or p38 MAP kinases[15,16]. Therefore, TAK1 MAP3K may phosphorylate both MAP2K and the MAP3K-like kinase NIK (Fig. 6). A conserved threonine residue, Thr 559, in NIK that is important for its catalytic activity[23] is not phosphorylated by TAK1 but is autophosphorylated[23]. Our preliminary results indicate that TAK1 may phosphorylate the N-terminal region of NIK (data not shown), its non-catalytic domain, which might partially activate NIK so that it can autophosphorylate in the catalytic domain, possibly at Thr 559, and stimulate catalytic activity. Our results should enhance understanding of the IL-1 signalling pathway. □

Methods

Expression vectors and antibodies. To overexpress TAK1(K63W), we constructed the expression vector pCMV-TAK1(K63W), encoding TAK1(K63W) under the control of the cytomegalovirus (CMV) promoter. Mammalian expression vectors encoding Myc–NIK, Myc-NIK(KK429-4309AA), Flag–IKK-α, Flag–IKK-α(K44A), HA–JNK, TAK1, TAB1 and HA–TAK1 have been described[3,8,17]. The NIK mutant, NIK(STT549,552, 559AAA), was generated by overlapping PCR. Polyclonal rabbit antibodies against TAK1 and TAB1 were produced against peptides corresponding to amino acids 554–579 of TAK1 and amino acids 480–500 of TAB1, respectively.

NF-κB-dependent reporter assays. 293 cells (1.6×10^5 cells per well) were

seeded into 6-well (35-mm) plates. Cells were transfected at 2 days after seeding by the calcium phosphate precipitation method with an Ig-κ–luciferase reporter gene plasmid and each expression vector. The total DNA concentration (1.7 μg) was kept constant by supplementing with empty vector. Luciferase activity was determined using the Luciferase Assay System (Promega). A β-galactosidase vector (0.1 μg) under the control of the β-actin promoter was used for normalizing transfection efficiencies.

Protein phosphatase treatment. Cells were lysed in 1% NP-40 buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1 mM PMSF without phosphatase inhibitors. Aliquots of lysates were incubated with or without Lambda Protein Phosphatase (New England Biolabs) in phosphatase buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 0.01% Brij-35 and 2 mM $MnCl_2$ at 30 °C for 30 min.

***In vitro* phosphorylation assays.** To construct GST–IκBN, a cDNA encoding the first 72 amino acids of human IκB-α was subcloned into pGEX2T (Pharmacia). To construct GST–IκBN(S32A,S36A), Ser 32 and Ser 36 were changed to alanine by site-directed mutagenesis. Endogenous IKK-α was immunoprecipitated from 293 cells. Aliquots of immunoprecipitates were incubated with 5 μg bacterially expressed GST–IκBN or GST–IκBN (S32A,S36A) proteins in 15 μl kinase buffer containing 20 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 0.4 mM PMSF, 1 mM ATP, 20 mM creatine phosphate and 5 μCi [γ-^{32}P]ATP at 37 °C for 30 min. Samples were resolved by SDS–PAGE, and phosphorylated GST–IκBN was visualized by autoradiography.

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NF-AT activation requires suppression of Crm1-dependent export by calcineurin

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Nuclear import of the NF-AT transcription factors during T-cell activation requires the calcium-activated phosphatase calcineurin, which unmasks nuclear-location signals on NF-AT (refs 1–5). We show here that the nuclear import of NF-ATs is not sufficient to activate NF-AT target genes, as NF-ATs are subject to a futile cycling across the nuclear envelope owing to engagement with the exportin protein Crm1 (refs 6–8). Calcineurin suppresses this futile cycling by a non-catalytic mechanism involving the masking of nuclear export signals on NF-AT targeted by Crm1. This clustering of binding sites for calcineurin and Crm1 on NF-AT establishes an inherent competition between these molecules that imparts exquisite calcium sensitivity to the shuttling dynamics of the NF-AT transcription factors. Such a balance between nuclear import and export may regulate the action of other transcription factors.

The fungal metabolite leptomycin B is a specific inhibitor of the nuclear export signal receptor Crm1 (refs 6–12). Leptomycin B also inhibited NF-AT nuclear export following calcium ionophore-induced nuclear import (see Figs A–C in Supplementary Information). To assess the role of Crm1 in NF-AT4 shuttling dynamics, we investigated whether Crm1 overexpression would prevent NF-AT from moving to the nucleus. Overexpressed Crm1 had no effect on the cytoplasmic localization of NF-AT4 in resting cells, but completely blocked the nuclear localization of NF-AT4 in response to calcium ionophore (see Figs E, F in Supplementary Information). Similar results were obtained with NF-AT4(N), which lacks the Rel homology domain (see Figs D, F in Supplementary Information). We have shown previously that the nuclear-localization signal (NLS) on NF-AT is masked by the NLS-masking, or Z, domain[2], and that the NF-AT4(N)ΔZ mutant is constitutively nuclear (Fig. 1a). Significantly, overexpressed Crm1 efficiently exported NF-AT4(N)ΔZ from the nucleus (Fig. 1a), indicating that Crm1 actively exports NF-AT from the nucleus rather than interferes with its intramolecular NLS-masking mechanism.

To define regions of NF-AT4 required for Crm1-mediated export, we screened NF-AT(N)ΔZ mutants for those that could bypass Crm1 and remain in the nucleus. One mutant that lacked the amino terminus of NF-AT(N)ΔZ was exclusively nuclear, despite Crm1 overexpression (Fig. 1b). Two subdomains of this amino-terminal region were found to be sufficient for nuclear export of NF-AT4(N)ΔZ by Crm1 (Fig. 1b). We called these regions NES1 (amino acids 31–96) and NES2 (amino acids 99–154) to reflect the fact that each contained an autonomous NES sequence targeted by Crm1.

Crm1 has been shown to bind the nuclear-export signal (NES) of the Rev protein of human immunodeficiency virus (HIV)[7]. To determine whether the functionally defined NES sequences on NF-AT4 physically bind Crm1, we assayed interactions between the NF-AT mutants and Crm1. Crm1 displayed good affinity for a fusion protein consisting of glutathione *S*-transferase (GST) and NF-AT4(N), with no significant binding to GST (Fig. 1c). Deletion of the NES1 domain diminished, but did not abolish, Crm1 association. Similarly, a GST–NF-AT4(N) mutant lacking the